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Ammonia inhibition on *Arthrospira platensis* in relation to the initial biomass density and pH

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Abstract

In this study the combined effect of total ammoniacal nitrogen (TAN) concentration, initial biomass density and initial pH of the cultivation medium on growth of *A. platensis* was studied. The results indicate that TAN inhibition in relation to the initial biomass in unregulated pH cultures is neither a clearly biomass-independent nor biomass-dependent phenomenon. However, low biomass densities are more susceptible to ammonia inhibition than higher biomass densities. Higher biomass densities seems to mitigate ammonia inhibition through rapider assmimilation of TAN. In all cases studied the growth rates were lower compared to the cultures with nitrate as nitrogen source. It was observed that at low TAN concentration, although no ammonia inhibition occured the growth rates were decreased due to nitrogen limitation. Low TAN concentration triggered the accumulation of carbohydrates

affecting thus significantly the biomass composition. Ammonia losses from the cultivation system were also determined. Ammonia losses ranged between 17% and 80%.

Keywords: Ammonia inhibition; ammonia losses; biomass; microalgae; nutrients.

1. Introduction

Microalgal and cyanobacterial biomass production is of great interest because of its potential use in a series of industrial applications. Biofuels production using microalgal and cyanobacterial biomass is gaining interest and it is believed that it will contribute significant in the sector of renewable energy systems (Gouveia and Oliveira, 2009). However, nowadays, the microalgal cultivation for biofuels production is not economically feasible. It is pointed out that the use of wastewater streams improves the economic feasibility of such systems (Prajapati et al., 2013).

Some wastewaters and especially wastewaters from the agro-industrial sector, such as animal and poultry wastewaters are of great importance due to their rich content in nutrients, which can fulfill the microalgal nutrient needs (Prajapati et al., 2013). Among nutrients, nitrogen is one of the primary and essential macronutrients that microalgae need for their growth. Microalgae can utilize nitrogen from the surroundings in various forms, such as ammonia or nitrates. A sector of wastewaters of great interest is the effluents of the anaerobic digestion process, which contain high concentrations of nitrogen in form of TAN, that in some cases may reach over 10 g N l^{-1} (Yenigün and Demirel, 2013). However, it is well known that TAN above a concentration, which is microalgal/cyanobacterial species and culture pH dependent, inhibits growth of microalgae and cyanobacteria rendering the use of

these wastewaters problematic (Peccia et al., 2013). For example, *Arthrospira platensis*, which is one of the most resistant to ammonia-inhibition species, its growth was reported to be inhibited by 50% when ammonia concentration was about 140 mg l^{-1} at pH 10 (Belkin and Boussiba, 1991).

As far known, the main way by which ammonia inhibits microalgae and cyanobacteria is by poisoning their photosynthetic system (Abeliovich and Azov, 1976). More specifically ammonia seems to target the oxygen-evolving complex (OEC) causing photo-damage of Photosystem II (Dai et al., 2013; Drath et al., 2008). The degree of ammonia inhibition is a combination between TAN concentration and the difference in pH values (ΔpH) between cells and cultivation medium. The pH of the medium affects the degree of inhibition mainly by regulating the equilibrium concentration between ammonium (NH_4^+, the ionized form of dissolved ammonia) and free ammonia (NH_3, unionized form). Free ammonia (FA) is the main inhibitory form of TAN (Azov and Goldman, 1982; Boussiba, 1989; Boussiba and Gibson, 1991). Its inhibitory effect is due to its free penetration through the cell membranes. Because cells are unable to control the uptake of FA, the penetration into the cells results in its intracellular accumulation, acting toxic to the microorganisms (Drath et al., 2008). It was defined that free ammonia being a small gaseous molecule does not only penetrates freely into the cells from the surroundings, but also leaks freely from the cells into the surroundings (Boussiba and Gibson, 1991). Azov and Goldman (1982), mention that this FA's free penetration/leaking activity renders ammonia inhibition a biomass-independent phenomenon, because FA penetrates unregulated into the cells damaging them and then leaks to the medium again penetrating the next cell etc. Therefore the form of ammonia inhibition is not related to the biomass present in the culture but depends mainly on the concentration of ammonia (Azov

and Goldman, 1982). However, some consequently questions that rise are: what is the degree of TAN inhibition in relation to the biomass density present in the culture? And, can higher biomass concentrations mitigate the inhibitory effect of TAN by assimilating ammonia in rates which reduce its intracellular accumulation and therefore its inhibitory effect?

Since ammonia is present in wastewater, which could be applied for microalgal and cyanobacterial production, and since ammonium salts fertilizers are less expensive nitrogen source compared to other nitrogen (nitrates) fertilizers, scope of this study was to investigate the interaction of TAN concentration and biomass density (inoculum) in relation to the initial pH of the cultivation medium on the growth of *A. platensis* in pH-unregulated batch mode. It was hypothesized that the investigation of the interaction between biomass density and TAN concentration might lead to an optimum biomass to ammonia ratio that might be of interest in batch cultures as a strategy to address ammonia inhibition.

2. Materials and methods

2.1 Microorganism and culture conditions

The cyanobacterium *Arthrospira platensis* SAG 21.99 used in the study was obtained from SAG (Sammlung von Algenkulturen der Universität Göttingen). The inoculum for the experiments was prepared by the cultivation of *A. platensis* in Zarrouk under 60 μmol photons m^{-2} s^{-1} light illumination. As inoculum, cells in the exponential growth stage were used. The Zarrouk medium had the following composition (per l): 16.8 g $NaHCO_3$, 2.5 g $NaNO_3$, 0.5 g K_2HPO_4, 1.0 g K_2SO_4, 1.0 g NaCl, 0.04 g $CaCl_2$, 0.08 g Na_2EDTA, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$ and 1.0 ml of trace elements (per l): 2.86 g H_3BO_3, 0.02 g $(NH_4)_6Mo_7O_{24}$, 1.8 g $MnCl_2 \cdot 4H_2O$, 0.08 g Cu_2SO_4 and 0.22 g $ZnSO_4 \cdot 7H_2O$.

Experimental cultivations were carried out in 250 ml photobioreactors (PBR). The working volume was set on 150 ml. The cultures were aerated, in order to be agitated, with filtered air provided by a membrane air pump. The aeration rate was set low (around 0.1 $l l^{-1}$ min^{-1}), in order to prevent high ammonia stripping. The cultivation was carried out in air-conditioned room and culture temperature was kept constant at 30 (± 2) °C. Light intensity was set at 100 μmol photons m^{-2} s^{-1} (measured in the middle of the PBR) and was provided continuously through one 57W fluorescence tube-lamp on the one side of the PBR. Runs were carried out in duplicate.

2.2 Experimental design

A. platensis was cultivated in modified Zarrouk medium with five different TAN concentrations, replacing the nitrate nitrogen source ($NaNO_3$). Ammonia was added in the form of NH_4Cl. Each concentration had three different initial biomass densities (inoculum) and each combination of TAN concentration and biomass density was performed in three different initially adjusted pH values. The overall experiment design is shown in **Table 1**. Control cultures were also performed, in which *A. platensis* was cultivated in Zarrouk medium, with 2.5 g l^{-1} $NaNO_3$ as nitrogen source and initial biomass density of 125, 250 and 500 mg l^{-1}.

2.3 Analytical methods

Dry algal biomass was measured indirectly by spectrophotometry at 560nm as was described in a previous work (Markou et al., 2012). Proteins were determined according to the Lowry method (Lowry et al., 1951) using bovine albumin as standard. TAN was measured according

to Solorzano (1969). Carbohydrates were determined by the phenol-sulfuric acid method (DuBois et al., 1956) using D-glucose as standard. All biomass composition analyses were performed after the washing of the samples for several times with deionized H_2O. All spectrophotometric determinations were carried out on Cadas 30 (Dr. Lange, Germany) spectrophotometer and analyses were carried out at least in triplicates.

The growth rates were calculated according to the equation:

$$\mu = \frac{\ln B_{t1} - \ln B_{to}}{t}$$

Where, B_{t1}, B_{t0} is the actual and the initial biomass density (mg l^{-1}) and t is the duration of the cultivation (72 h). Relative growth rates refer to the ratio of the actual growth rate of a run to the maximum growth rate of the corresponding control with nitrates as the nitrogen source (μ μ_{max}^{-1}).

Ammonia losses were calculated as the difference between intracellular nitrogen (taken-up by cells) and the TAN removal. The intracellular nitrogen content was calculated based on the protein content divided by using the factor 5.95.Using this factor the intracellular nitrogen represents the total Kjeldahl nitrogen (i.e. organic nitrogen plus ammonia nitrogen) (González López et al., 2010). Ammonia losses are expressed as percentage of the initial total nitrogen added to the cultures. In the calculations the intracellular nitrogen content of the inoculum used was included.

3. Results and discussion

3.1 Effect of TAN concentration on growth rates of *A. platensis*

In this study, the combined effect of TAN concentration, initial biomass density (inoculum) and initial pH of the cultivation medium on growth of *A. platensis* was studied. As shown in **Figure 1**, the pH of the cultures increased in time and reached after 72 hours of cultivation values of 9.8 and above. It was observed that the increase in the initial biomass density resulted to higher final pH of the medium and that in general the pH values were proportional to the biomass density in the medium and the ammonia up-take and loss (**Figure 1**). It is known that during photosynthesis OH^- are generated and released to the medium, causing alkalization of the medium and consequently an increase in pH values occur (Shiraiwa et al., 1993). In contrast, ammonia up-take or loss by stripping, acidifies the medium because H^+ are generated forcing the pH to be decreased (Grobbelaar, 2004). However, in this study it was observed that the alkalization rate due to photosynthesis was higher than the acidification rate due to ammonia uptake and/or loss, resulting to a net increase in pH values.

As was mentioned before, the pH value of a culture determines the degree of the concentration of FA, which is the most toxic form of TAN. Because the cultures in the present study were performed in batch mode and because the pH was adjusted only in the beginning of the experiments (no regulation of pH was carried out during the cultivation), the ammonia inhibition effect was a dynamic process, changing over time. However, taking into consideration that the pH increased considerable in all of the cases investigated, it was considered that the growth reduction due to ammonia inhibition was the result mainly of the effect of ammonia during the first hours of cultivation, and therefore any results were

influenced mainly by the initial culture conditions (biomass density, pH and TAN concentration).

As shown in **Figure 1**, growth rates of *A. platensis* were steady or slightly increased when TAN concentration increased up to 100 mg N l^{-1}, while for TAN 150 and 200 mgN l^{-1} growth rates were significantly negatively affected. In combination with the increase of TAN concentration up to 100 mg N l^{-1}, the variation of the initial pH did not display significant effect on the various biomass densities investigated; however at TAN concentrations 150 mg N l^{-1} and higher, the increase of the initial pH had more severe negative effect on growth (**Figure 1**). More specifically, while in cultures with pH 8 and TAN of 200 mg N l^{-1} the growth was relative little affected, in cultures with pH 9 and pH 10 growth was absolutely inhibited (except in culture with pH 9 and 500 mg l^{-1} initial biomass density). At lower pH values in which the ammonium form dominates, the ammonia inhibition is lower because ammonium is unable to penetrate freely the cell membranes, and it is taken up actively by the cells by demand. Therefore its inhibition effect is significant lower than of FA (Abeliovich and Azov, 1976; Källqvist and Svenson, 2003).

At TAN concentration of 150 mg N l^{-1} and higher, the increase of the initial biomass density resulted in a lower inhibition. In **Figure 2**, some selected series of runs in which ammonia inhibition clearly occurred are illustrated. As shown in this figure, there were some cultures in which initial biomass concentration affected significantly the overall growth capability of *A. platensis.* Especially, in the cultures with high TAN concentration and high pH, increasing biomass density resulted in the mitigation of ammonia inhibition. This positive effect however, was observed only between runs with 125 and 250 mg l^{-1} of initial biomass, while the differences between runs with 250 and 500 mg l^{-1} were low or even negligible.

These results indicate that the ammonia inhibition regarding the relationship between initial biomass density and TAN concentration in unregulated pH cultures is not a clearly biomass-independent phenomenon as was suggested by Azov and Goldman (1982). However, as shown in (**Figure 3**) the ammonia inhibition is neither a clear biomass-dependent phenomenon.

As was mention before, according to Azov and Goldman (1982) the free penetration/leaking activity of FA is the reason that renders ammonia inhibition a biomass-independent phenomenon. However, the results of the present study suggest that higher biomass density could assimilate TAN, mitigating the ammonia inhibition effect. This perhaps shows that some critical ratio of biomass density to TAN concentration exists, in which the inhibition is biomass dependent phenomenon. Taking into consideration the results of this study, low biomass densities are more susceptible to ammonia inhibition than higher biomass densities. Still, a clear relationship between ammonia inhibition and biomass density cannot be drawn by the pH un-regulated batch mode cultures. Therefore pH-stat cultures with steady state conditions (pH and biomass density) should be employed to gather more appropriate results.

Nevertheless, in all cases, even at the lowest TAN concentration the growth rates (for 72 hours of cultivation) of *A. platensis* were more than 15% lower than the growth rates of the runs with $NaNO_3$ as nitrogen source (served as control runs). Although ammonia is often preferred to be taken up by microalgae because it can be directly assimilated in amino acids while nitrate first needs to be reduced to ammonia (Boussiba and Gibson, 1991; Graham and Wilcox, 2000), it is often reported that microalgal and cyanobacterial growth rates are lower compared to cultures with nitrate as nitrogen source (Belkin and Boussiba, 1991; Kim et al.,

2013; Lin et al., 2007). Regarding the species of *A. platensis*, Belkin and Boussiba (1991) reported that TAN concentration of 70 mg l^{-1} and 140 mg l^{-1} caused a decrease in its photosynthetic capacity of 30% and 50%, respectively. Moreover, Converti et al. (2006) reported that *A. platensis* was negatively affected even when TAN concentration increased from 30 mgN l^{-1} and above. However, in this study the reduced growth rates at the lower TAN concentration were not due to ammonia inhibition but due to the growth hindering caused by nitrogen limitation (see section **3.3**).

Considering the importance of ammonia as nitrogen source for microalgal and cyanobacterial biomass production, using either wastewater streams or ammonium salts fertilizers (Bezerra et al., 2008; Carvalho et al., 2004; Olguín et al., 2003; Soletto et al., 2005; Yuan et al., 2011), the inhibition of ammonia contained in wastewater is an issue that should be addressed. To overcome ammonia inhibition the main strategies that are suggested by other researchers are: (a) dilution of wastewater to render the ammonia concentration non-inhibitory to microalgae (He et al., 2013), (b)) regulation of the pH to the lowest possible values so that free ammonia is converted to the non-toxic ammonium ion (Azov and Goldman, 1982) and/or (c) using a cultivation mode (continuous, semi-continuous or fed-batch) so that ammonia will be added in non-inhibitory concentrations (Ganuza et al., 2008; Soletto et al., 2005; Yuan et al., 2011). However, it can be concluded from the present study that in batch cultures higher initial biomass densities could be also a strategy to address, at least the detrimental effect of ammonia on *A. platensis*.

3.2 Ammonia uptake and ammonia losses

Ammonia analyses after 72 hours of cultivation showed that in the most runs ammonia was absolutely removed except in some runs with initial TAN concetration of 150 mg l^{-1} (**Figure 4**). However, the removal of ammonia was not only due to its up-take from *A. platensis* but also due to stripping and its loss to the atmosphere. Therefore, the estimation of ammonia losses was made. Ammonia losses were calculated as the difference between intracellular nitrogen (taken-up by cells) and the total ammonia removal. As shown in **Figure 5**, three general observations could be made, namely that (a) ammonia losses were lower as initial biomass density increased, (b) ammonia losses were higher as TAN concentrations increased and (c) ammonia losses were the highest when initial pH was 10. These general observations were expected. For the first observation an explanations is that since higher initial biomass densities (inoculum) lead to higher final biomass densities (data not shown) the losses were lower due to higher uptake and assimilation of ammonia. The second observation is due to the fact that when more ammonia is added to the cultures, the growth inhibition intensifies causing a decrease of ammonia up-take. Consequently higher amounts of TAN are more susceptible to be stripped and lost. The third observation is due to the fact that as pH increases the dissociation equilibrium of dissolved ammonia favors the formation of the gaseous (NH_3) ammonia which is easier stripped than the protonated ammonium (NH_4^+) form which dominates in lower pH values (pK=9.25).

It has been pointed out that ammonia stripping and loss to the atmosphere may be the most important mechanism of ammonia removal when microalgae or cyanobacteria are used for nutrient removal from wastewaters (Olguín et al., 2003). In the present study ammonia losses ranged from about 17% to about 80%. These results suggest that in cultures of the alkalophilic *A. platensis*, the ammonia application is highly susceptible to great losses.

However, the high ammonia losses which were observed in this study may be also due to the type of agitation (aeration) used. Agitation by aeration results to more ammonia losses in comparisson to agitation by stirring (Pouliot et al., 1989).

3.3 Biomass composition

In **Figure 6**, the variation of the biomass composition of *A. platensis* in time is ilustrated. As shown, TAN concentration affected significantly the biomass composition. In general, it was observed that in low TAN concentrations *A. platensis* was rich in carbohydrates (about 50-60%) and poor in proteins, a fact that indicates that these cultures were nitrogen limited. It is well known that *A. platensis* when it is nutrient limited, such as in nitrogen or phosphorus, it accumulates carbohydrates in its biomass (Markou et al., 2012; Olguín et al., 2001). The graduall increase in TAN concentration, resulted in a lowering of the nitrogen limitation effect and hence the carbohydrate content decreased, while protein content increased. At TAN concentration of 100 mg l^{-1} and above *A. platensis* reached almost its typical biomass composition, suggesting perhaps that the ammonia inhibition by itself does not result to the alteration of the biomass composition. In cultures with pH 10 the carbohydrate content was higher than in pH 8 and pH 9 probably due to the higher ammonia losses by stripping (see section **3.2**).

The results indicate that, although low ammonia concentration cause lower growth inhibition, it is possible to cause nitrogen limitation with a consequence effect on the biomass composition and probably on growth rates because of the nutrient limitation. However, low TAN concentrations could be a strategy of choise to produce carbohydrate-rich microalgal

biomass, which could be used for the production of bioethanol as biofuel (Markou et al., 2013; Miranda et al., 2012).

4 Conclusions

In the present study the effect of ammonia inhibition in relation to the initial biomass density (inoculum) and pH of the cultivation medium on the growth of the alkalophilic cyanobacterium *A. platensis* was investigated. The results suggest that low biomass densities are more susceptible to ammonia inhibition than higher biomass densities., while higher biomass densities seems to mitigate ammonia inhibition through rapider assmimilation of TAN. However, there is no a clear relationship between biomass density and ammonia inhibition. At low TAN concentrations the cultures were nitrogen limited resulting to an alteration of the biomass composition. Ammonia inhibition by itself does not result to the alteration of the biomass composition. Increasing TAN concentration, however resulted to an increase of the degree of ammonia inhibition. High ammonia concentrations also resulted in large ammonia losses to the atmosphere, which reached almost 80% at the highest TAN concentation and highest pH values.

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Figure and Table Captions

Figure 1. Left row: Final pH values (after 72 h of cultivation) of *A. platensis* (a) pH 8, (b) pH 9 and (c) pH 10. Right row: relative growth rates (as ratio of the control) of *A. platensis*.

Figure 2. Growth rates (as % of the control) of *A. platensis* cultivated under various pH and TAN concentations. ●: 9/100, ○: 10/100, Δ: 8/150, ■: 9/150, ▼:10/150, □: 8/200. Symbols x/y mean pH/TAN concentration.

Figure 3. Growth rates (as ratio to the control growth rates) of *A. platensis* cultivated in various TAN concentations and initial biomass densities and different pH. ●: 125 mg l^{-1}, ○: 250 mg l^{-1} and ▼: 500 mg l^{-1}

Figure 4. Remove of ammonia from batch cultures of *A. platensis* cultivated with various ammonia concentrations and initial biomass densities.

Figure 5. Ammonia losses from batch cultures of *A. platensis* cultivated with various ammonia concentrations and initial biomass densities. ●: 50 mg NH_3-N l^{-1}, ○: 75 mg NH_3-N l^{-1}, ▼: 100 mg NH_3-N l^{-1} and Δ: 150 mg NH_3-N l^{-1}.

Figure 6. Biomass composition of *A. platensis* cultivated in batch mode with different ammonia concentrations and initial biomass densities with three different initial pH values (a) pH 8, (b) pH 9 and (c) pH 10. Left row: carbohydrates content. Right row: Proteins content.

Table 1. Experimental design.

Figure 1

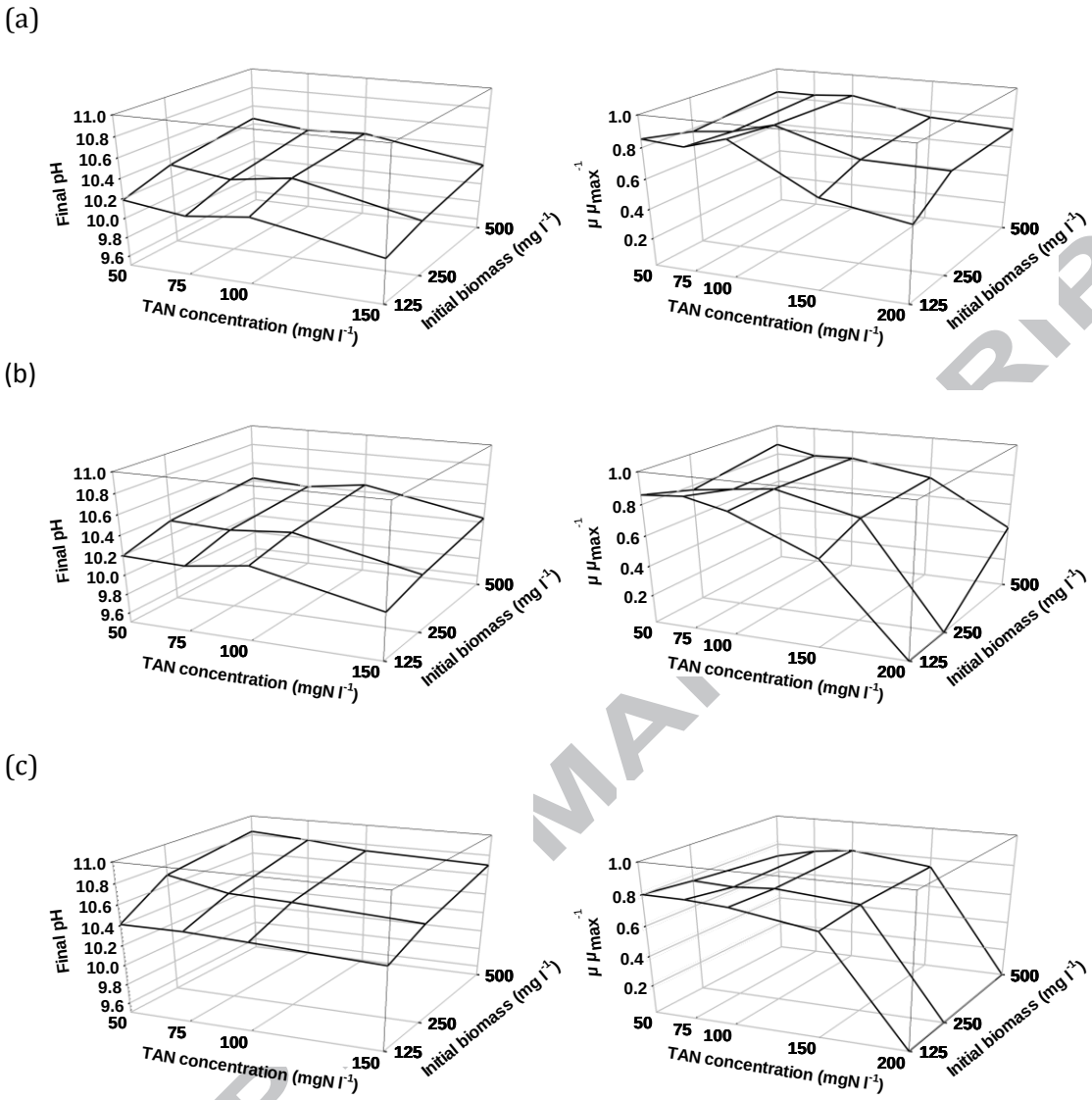

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Figure 2

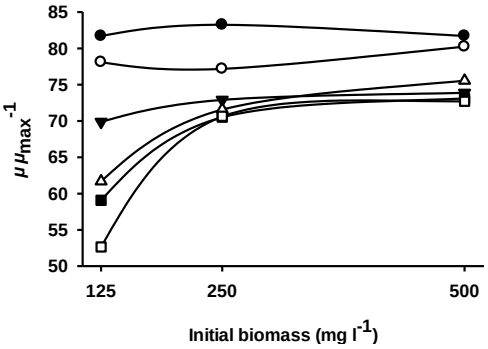

Figure 2. Growth rates (as % of the control) of *A. platensis* cultivated under various pH and TAN concentations. ●: 9/100, ○: 10/100, Δ: 8/150, ▉: 9/150, ▼:10/150, □: 8/200. Symbols x/y mean pH/TAN concentration.

Figure 3

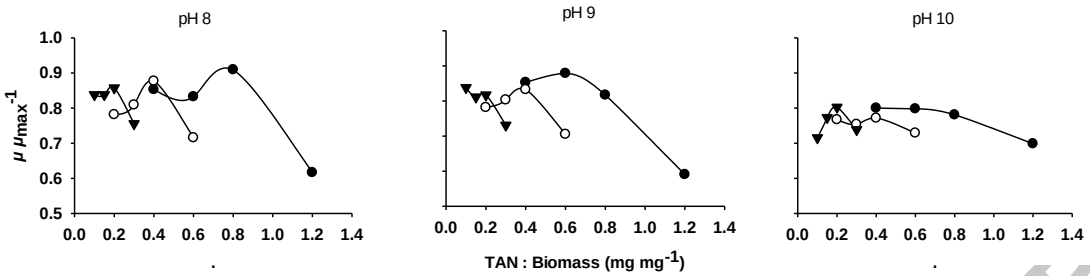

Figure 3. Growth rates (as ratio to the control growth rates) of *A. platensis* cultivated in various TAN concentations and initial biomass densities and different pH. ●: 125 mg l^{-1}, ○: 250 mg l^{-1} and ▼: 500 mg l^{-1}

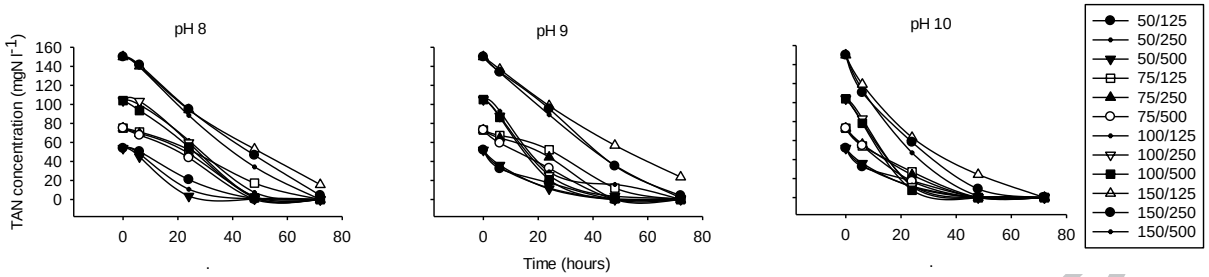

Figure 4. Remove of ammonia from batch cultures of *A. platensis* cultivated with various ammonia concentrations and initial biomass densities.

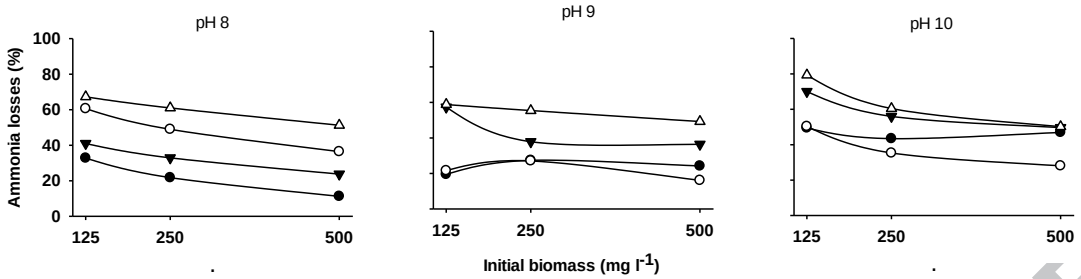

Figure 5. Ammonia losses from batch cultures of *A. platensis* cultivated with various ammonia concentrations and initial biomass densities. ●: 50 mg NH_3-N l^{-1}, ○: 75 mg NH_3-N l^{-1}, ▼: 100 mg NH_3-N l^{-1} and Δ: 150 mg NH_3-N l^{-1}.

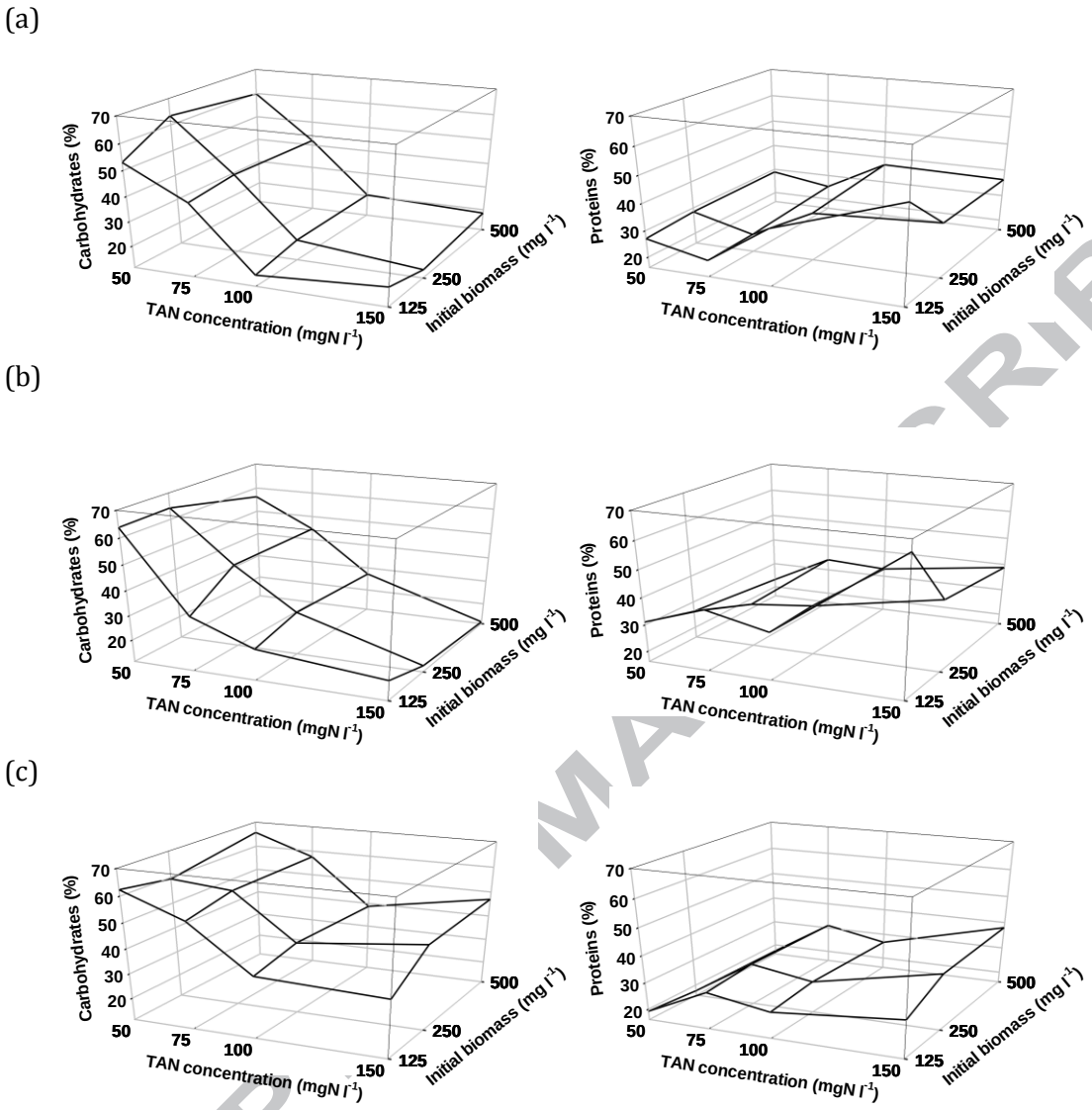

Figure 6. Biomass composition of *A. platensis* cultivated in batch mode with different ammonia concentrations and initial biomass densities with three different initial pH values (a) pH 8, (b) pH 9 and (c) pH 10. Left row: carbohydrates content. Right row: Proteins content.

Table 1. Experimental design.

Initial pH	Biomass density (mg l^{-1})	Total ammonia nitrogen concentration (mg l^{-1})				
8	125 250 500	50	75	100	150	200
9	125 250 500	50	75	100	150	200
10	125 250 500	50	75	100	150	200

Highlight

- Arthrospira growth with ammonia as nitrogen source was performed
- The effect of NH_3 concentration, initial biomass density and initial pH was studied
- NH_3 inhibition is neither a biomass-independent nor biomass-dependent phenomenon
- In low NH_3 concentration growth rates decreased due to nitrogen limitation.
- NH_3 stripping and losses were high as 80%.